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# Biochemical markers of type II collagen breakdown and synthesis are positioned at specific sites in human osteoarthritic knee cartilage

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## Summary

*Objective*: To investigate whether type II collagen turnover markers used for osteoarthritis (OA) activity evaluation in body fluids can be detected at the level of specific histological features of OA cartilage tissue, as well as how they relate with each other at this level.

*Methods*: Adjacent sections were obtained from full-depth cartilage biopsies from 32 OA knees. Immunohistochemistry was performed for Helix-II and CTX-II, which are type II collagen fragments originating from the triple helix and the telopeptide region, respectively, and believed to reflect distinct breakdown events, as well as for type IIA N propeptide (PIIANP), a biochemical marker reflecting synthesis of type IIA collagen.

*Results*: Helix-II and CTX-II were detected in areas where collagen damage was reported previously, most frequently around chondrocytes, but also frequently in regions not previously investigated such as the margin area and close to subchondral bone, including vascularization sites and bone–cartilage interface. The latter is CTX-II's prevailing position and shows rarely Helix-II. PIIANP co-localized with Helix-II and CTX-II on a limited number of features, mainly in deep zone cartilage. Overall, our analysis highlights clear patterns of association of the markers with specific histological features, and shows that they spread to these features in an ordered way.

*Conclusion*: Helix-II and CTX-II show to some degree differential selectivity for specific features in cartilage tissue. CTX-II detection close to bone may be relevant to the possible role of subchondral bone in OA. The restricted co-localization of breakdown markers and PIIANP suggests that collagen fragments can result only partially from newly synthesized collagen. Our study strengthens the interest for the question whether combining several markers reflecting different regional cartilage contributions or metabolic processes should allow a broader detection of OA activity.


*Key words*: Helix-II, CTX-II, PIIANP, Cartilage turnover, Osteoarthritis.

## Introduction

Osteoarthritis (OA) is a very common joint disease in the western world. It is characterized by loss of articular cartilage accompanied to some degree by synthesis of matrix molecules. These processes result in morphological changes, malfunction of the joint and morbidity. Changes occur in all the regions of articular cartilage, and are showing up in a variety of ways, used for grading the disease[1]. The cell responsible for elaborating and maintaining the cartilage matrix is the chondrocyte, which therefore responds specifically to the local needs by performing both catabolic and anabolic activities. The reason why the chondrocytes fail to comply with these tasks in OA is not understood, and there is presently no therapy targeting chondrocytes and preventing OA. Much information was, however, gained on the molecular changes occurring in the cartilage matrix during OA development[2].

The cartilage matrix is composed mainly of proteoglycans and type II collagen fibrils. It is proposed that damage to the collagen network leads to loss of tensile strength, and is a critical event in OA[2,3]. An elegant approach for assessing this damage was based on immunoreactive probes recognizing neoepitopes in the unwound triple helix of the collagen molecule[2,4–7]. This approach showed a correlation between unwound collagen and collagen/proteoglycan content of cartilage, and it also showed that this collagen damage starts at the articular surface and around the chondrocytes, and then progresses to deeper zones. These probes did not detect collagen damage in areas close to the subchondral bone, despite the possible role of subchondral bone in OA[8,9]. Interestingly, these probes have all in common that they recognize damage initiated by collagenases[6], which are proteinases able to cleave the native triple helix of collagen. This property of these probes, together with their co-localization with collagenases in histological sections[10] and the inhibition of collagen damage upon treatment with collagenase inhibitors[11,12] led to the view that collagenases may play a key role in OA. On the other

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hand, however, inhibition by these collagenase inhibitors was often partial, suggesting that other proteinases may contribute to collagen cleavage[11]. Accordingly, chondrocytes and cells of other tissues of the joint were reported to express proteinases with different cleavage specificities toward collagen compared with collagenases, and able to attack collagen at the level of its telopeptides or of destabilized triple helices[13–17]. These included matrix metalloproteinases, such as MMP-9 and MMP-3, and a series of cysteine dependent cathepsins, such as cathepsin K, B, L, and S. To our knowledge, human articular cartilage sections have not yet been probed for collagen breakdown by these other proteinases, and possible damage to the collagen network due to these proteinases has not been identified within the cartilage joint.

A series of studies of disease activity in OA are based on the urinary levels of CTX-II, a fragment arising from the telopeptide region of type II collagen, and that can be generated by the action of several of the non-collagenase proteinases mentioned above[13,18,19]. In patients with knee or hip OA, elevated urinary levels of CTX-II correlated with radiographic scores including joint space narrowing, joint surface, joint pain, joint inflammation, and bone sclerosis[20–23]. Furthermore, CTX-II levels responded to a number of factors/conditions considered to affect specifically bone, such as bisphosphonates, calcitonin, selective estrogen receptor modulators (SERMs), or postmenopausal status[19,24–27] thereby reflecting possibly to some degree the suspected bone–cartilage interactions in OA development[8]. In contrast, the menopausal status did not induce increased release of fragments arising from the helix part of type II collagen into body fluids[2,28]. One of them, Helix-II, was compared to CTX-II in the same OA patients, and despite the fact that both were elevated they correlated only weakly with each other[14,29]. This suggests that Helix-II and CTX-II reflect distinct biological/enzymatic pathways of cartilage collagen degradation. The question of the association of CTX-II and Helix-II with distinct events in cartilage tissue remained, however, to be investigated.

Finally, several studies addressed the question of collagen synthesis in articular cartilage. *In situ* hybridization and immunohistochemistry of type II collagen propeptides showed intense focal staining in OA cartilage, particularly away from the areas showing damaged matrix, whereas collagen synthesis was not detected in cartilage from healthy patients[30–32]. Both the C and N propeptides showed similar results. Of particular interest is that the N propeptide corresponds to the fetal splice variant, type IIA N propeptide (PIIANP), indicating the potential reversion of the cells to a chondroprogenitor phenotype possibly aiming at matrix repair[32]. However, serum levels of PIIANP and of the C-terminal propeptide, PIICP, were decreased in OA patients compared with healthy controls[31,33,34], thereby showing an apparent discrepancy with the observations at the tissue level. Furthermore, decreased levels of PIIANP corresponded with increased levels of CTX-II monitored in the same study, and were therefore interpreted as uncoupling of collagen type II synthesis and degradation[33]. The question of the inter-relation of collagen synthesis and breakdown at the level of the same cartilage feature has, however, not been addressed.

There is thus insufficient knowledge on the association of possibly different collagen breakdown mechanisms with distinct OA events, as well as on their relation with collagen synthesis. Furthermore, it has insufficiently been investigated which specific tissue alterations are reflected by clinical markers of collagen turnover measured in body fluids. In the present study, we probe full-depth sections of OA cartilage with clinical markers of (1) type II collagen degradation arising from the helix domain, Helix-II; (2) from the telopeptide domain, CTX-II; and (3) of collagen synthesis, and analyzed their association with specific histological features. This analysis demonstrates clear patterns of association/dissociation of each of these markers with each other, and with OA histological events.

## Materials and methods

### PATIENTS

Cartilage samples from 50 OA knees were collected from patients undergoing total knee replacement according to an approved clinical protocol. Briefly, all patients were postmenopausal women [mean age 65.3 years (11.2 standard deviation [SD])] who had not received hormone replacement therapy; were not diagnosed for any other endocrinological, metabolic or bone-related disorders; were not severely overweight (body mass index [BMI] $< 35$); had less than 4 h of physical activity per week. Collection of tissue specimens was approved by the Danish ethical committee, and all OA patients signed an informed consent formula. All patients were diagnosed with total knee OA. The degree of OA was graded by experienced orthopedic surgeons prior to surgery by Ahlbäck's X-ray classification[35] and postsurgery by visual Outerbridge scoring[36]. All patients included in the study were graded an Ahlbäck score between 3 and 5 for and an Outerbridge score of 3 or 4 of the worst affected position of knee.

### TISSUE PREPARATION

Knee discards were recovered immediately after surgery in sterile conditions. Biopsies were cut from both lateral and medial sides of femur condyles and tibia plateau including the loading zone, as well as the margin zone whenever possible (Fig. 1). All biopsies were fixed in 10% buffered formalin for 48 h at room temperature (RT) and decalcified in 0.2 M Idranal® III solution (Riedel-van Häen, Sigma–Aldrich, Denmark) under constant agitation at RT for up to 4 weeks depending on the biopsy. They were then processed according to standard pathological procedures, embedded in paraffin, and cut longitudinally so as to show full-depth cartilage from the joint surface to subchondral bone, as well as whole area from the loading toward the marginal zone (Fig. 1). Adjacent sections (5 μm) were obtained and mounted on positive Superfrost® glass slides (Hounisen, Denmark).

### HISTOPATHOLOGICAL GRADING

Sections were deparaffinized, hydrated and one section from each patient was stained with an optimized fast green–Safranin O (FGSO) trichrome staining protocol[37,38]. Briefly, sections were incubated for 10 min in Mayer's acidic hematoxylin (Sigma–Aldrich, Denmark), rinsed in tap water, incubated for 10 min in 0.001% Fast Green FCF (Sigma–Aldrich, Denmark) solution, rinsed in 1% acetic acid and incubated 20 min in 0.1% Safranin O (Sigma–Aldrich, Denmark) solution. Next the slides were rinsed and dehydrated in a series of distilled water, alcohols and xylene, and mounted in Pertex mounting medium (Sakura Prohosp, Denmark). All biopsies were graded according to the Mankin score[38,39]. Biopsies scoring middle or late phase (Mankin 4–10) OA were included in the study, while biopsies scoring early (Mankin $< 4$) or terminal phase (Mankin $> 10$) OA were excluded, because of lack of OA features of interest. In this way, a total of 58 biopsies were analyzed (13 lateral femur, 13 medial femur, 18 lateral tibia, and 14 medial tibia biopsies). These biopsies correspond to 32 knees, 26 of which were from patients diagnosed with primary OA, and six of which had been subjected to injuries including menisectomy, fracture, and rupture of the cruciate ligament. The OA type of each biopsy is shown in Fig. 4.

### IMMUNOHISTOCHEMISTRY

For immunodetection of Helix-II, we used the polyclonal rabbit antibody which recognizes the ERGETGPhypGTS sequence from the helical region and was previously described[14]. For immunodetection of PIIANP we used the polyclonal rabbit antibody which has been previously described and used in immunochemistry experiments[32,40,41]. For immunodetection of CTX-II we used a new polyclonal rabbit antibody raised against the six amino-acid sequence (EKGPDP) of the C telopeptide of type II collagen. Competition experiments using synthetic peptides show that the antibody recognizes specifically this amino-acid sequence and the recognition is dependent on the presence of a proline at the C-terminal end. This antibody does not recognize un-cleaved type II collagen, or homologous sequences

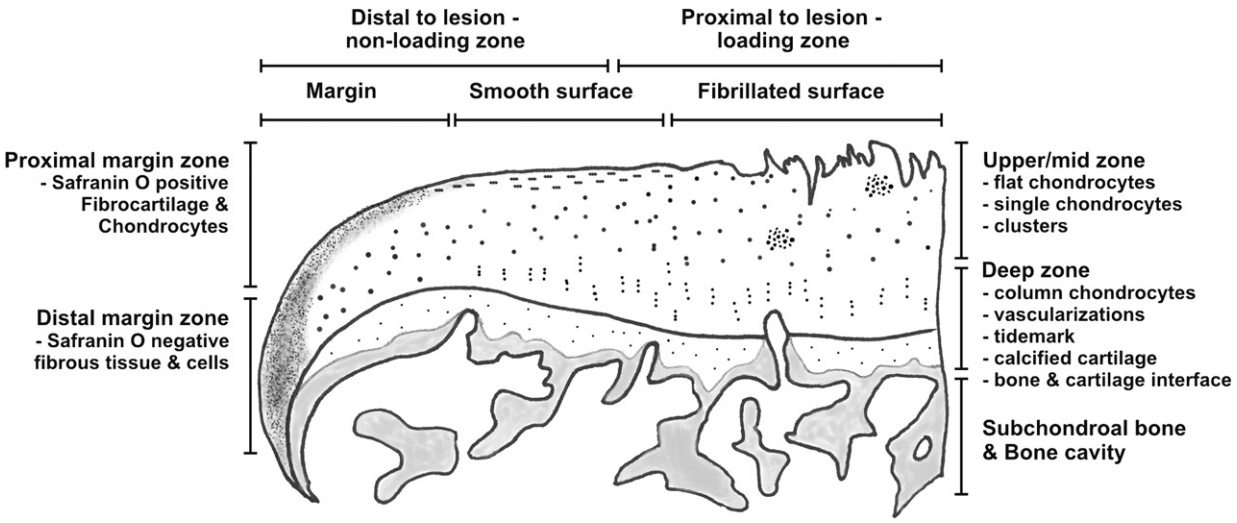


Fig. 1. Schematic view of a typical biopsy section used in the present study. The figure is aiming at defining the distinct areas and histological features of OA cartilage, mentioned in the present study. Note that ''flat chondrocytes'' are positioned close to the surface of the joint. ''Column chondrocytes'' appear in stacked lacunas in the deep zone above the tidemark. Vascularization appears in deep zone cartilage areas, as invasion into calcified cartilage and even further through the tidemark.

from type I and III collagens. The demasking procedure and antibody concentrations were optimized to give optimal visualization of the antigen, and no background signals as evaluated by using pre-immune serum at the same concentration. Staining with Helix-II and PIIANP followed the same procedure and was based on typical peroxidase coloring and tyramide enhancement. Sections were deparaffinized, blocked for endogen peroxidase with $H_2O_2$ for 30 min in 99% ethanol and then hydrated. Sections were then demasked by overnight incubation (8–13 h) at 63°C in citrate buffer (pH 6.0) or for 2 h in Tris–ethylenediamine tetraacetic acid buffer (pH 9.0) at 80°C. Sections were blocked in 0.5% casein and for endogenous biotin according to DAKO (Denmark). Sections were then incubated for 2 h at RT with antibody against Helix-II (diluted 1:4000), or CTX-II (diluted 1:3000), or PIIANP (diluted 1:1500). This incubation was followed by a 30-min incubation with horseradish peroxidase (HRP) conjugated antirabbit polymer (EnVision+, DAKO, Denmark). Sections were tyramid-enhanced by incubating for 10 min with biotinylated tyramide and for 30 min with HRP conjugated streptavidin. Development was performed using diaminobenzidine tetrahydrochloride (DAB+) (DAKO, Denmark), enhanced with $CuSO_4$. Finally the sections were counter-stained with Mayer's acidic hematoxylin, dehydrated, and mounted in Pertex. Digital pictures were obtained using a computer assisted microscope and only modified by enhancing the contrast and light; no background was removed or altered.

### SCORING OF IMMUNOSTAININGS AND STATISTICS

Eight adjacent sections were taken from each biopsy. Two were stained with FGSO in order to identify histological areas of interest, while the others were immunostained for CTX-II, Helix-II, and PIIANP, in duplicate to assess reproducibility. They were analyzed with a 10× or 20× objective. The number of biopsies where given histological features were identified was counted. For each feature, the presence or absence of the respective markers was recorded. Only clear signals as shown in Fig. 2 were taken into account. The frequency of biopsies showing an association of the respective markers with each of the features was determined. Furthermore, the statistical significance of co-localizations of markers was analyzed by Fisher's exact test, testing whether the detection of a given marker increased significantly the chance to detect also another marker.

## Results

### TYPICAL FEATURES APPEARING UPON STAINING WITH FGSO AND ANTIBODIES AGAINST COLLAGEN TURNOVER MARKERS

Upon FGSO staining, the biopsies showed well-known characteristics of OA cartilage (Fig. 1), including loss of proteoglycan in the upper and midzone cartilages [Fig. 2(A,B)], fibrillated surfaces [Fig. 2(B)], clustered chondrocytes [Fig. 2(C)], and vascular invasions [Fig. 2(D)]. Actually, one or several of these features were present in at least 90% of the biopsies (Fig. 3). These biopsies showed also areas without morphological damage, such as deep zone and margin area cartilage [Fig. 2(E)]. Upon immunostaining, the biopsies showed immunoreactivity for the collagen degradation markers, Helix-II and CTX-II, as well as for the collagen synthesis marker, PIIANP, which all are used to assess OA through measurements in body fluids. Figure 2 shows typical examples of how these immunoreactivities appear, whether associated with chondrocytes [Fig. 2(A–F)] or with matrix [Fig. 2(B,D,F)], or occurring close to [Fig. 2(B–D)] or away from lesions [Fig. 2(E,F)]. Because of the heterogeneity of OA, and also because immunostaining intensity is not a reliable quantification method, we chose to analyze these immunostainings by determining their frequency of association with given histological features.

### OVERALL ANALYSIS OF THE DISTRIBUTION OF COLLAGEN TURNOVER MARKERS

Figure 3 gives an overview of how frequently Helix-II, CTX-II, or PIIANP immunoreactivity was detected in the different cartilage areas. These immunoreactivities were widely distributed over the different regions, including deep zone cartilage and margin cartilage, i.e., regions appearing non-damaged, and located sometimes far away from where lesions are seen. They also showed a clear pattern of association of immunoreactivity with specific histological features of these cartilage regions. Immunoreactivities were associated most frequently with chondrocytes (in 60–80% of the biopsies in the case of Helix-II), and this holds true whether the chondrocytes are positioned in the upper, middle, deep, or lateral zone, and whether they are organized in single lacunae, in clusters, or in columns. In contrast, immunoreactivity is more rarely detected in interterritorial matrix, whether in the mid or deep zone and whether calcified or not, and despite frequent evidence for loss of proteoglycan in the midzone. However, matrix associated immunoreactivity appeared quite frequently in specialized areas of the matrix, such as superficial matrix, fibrillated matrix, margin matrix, the bone–cartilage interface, and sites of vascularization. As

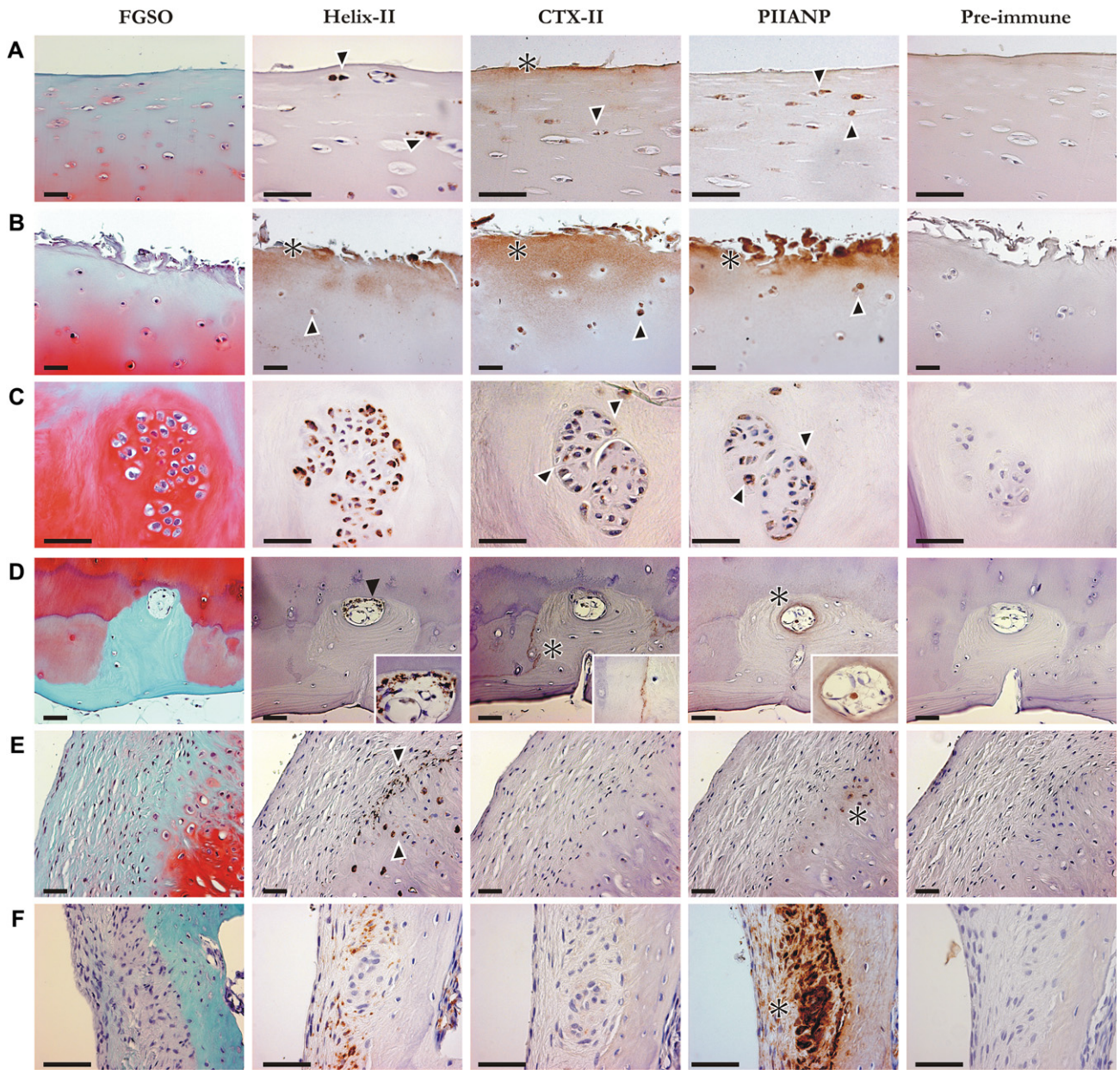


Fig. 2. Examples of histological appearance and immunoreactivity of Helix-II, CTX-II and PIIANP in different areas of OA cartilage. Different areas of OA cartilage (rows A–G) were stained as indicated (columns I–V). (A) Superficial area. Toward the surface chondrocytes are flat, and Safranin O staining (red) is absent, indicating loss of proteoglycan. Flat chondrocytes are positive for Helix-II, CTX-II and PIIANP (arrowheads). Note that the interterritorial matrix does not show any immunoreactivity despite the loss of proteoglycan, but surface is positive for CTX-II. (B) Fibrillated superficial area. Absence of Safranin O in the fibrillated zone indicates loss of proteoglycan. The fibrillated (but not the underlying) matrix and most chondrocytes are positive for Helix-II, CTX-II, and PIIANP. (C) Midzone area showing clonal chondrocytes and Safranin O stained proteoglycan (red). Most clonal chondrocytes show Helix-II and some show CTX-II and PIIANP. The matrix does not show any immunoreactivity. (D) Deep zone area is shown in five adjacent sections. Note proteoglycan-containing un-calcified and calcified cartilage (dark and light red, respectively), their tidemark separation (purple line), the subchondral bone (blue–green), and vascular invasion (asterisk in FGSO). The latter shows Helix-II and PIIANP (inset at higher magnification), whereas the bone–cartilage interface shows CTX-II and no Helix-II and PIIANP (asterisk and inset in CTX-II). The interterritorial matrix does not show any immunoreactivity. (E) Proximal margin. Safranin O positive fibrocartilage (red) and fibrous tissue (blue–green). Chondrocytes show Helix-II and weak PIIANP immunoreactivity. Their levels are a bit higher at the cartilage–fibrous tissue interface. The proximal margin area is devoid of CTX-II. (F) Distal margin. Note fibrous tissue (grayish blue) with numerous cells, and bone (blue–green). The cells show high levels of Helix-II and PIIANP. The latter is also abundant at the interface between bone and fibrous tissue. The distal margin area is devoid of CTX-II. Control sections adjacent to the one incubated anti-CTX-II (A,C) and Helix-II (B–F) were incubated with corresponding pre-immune antibody and did not show immunoreactive signals. Bars: 50 μm.

a matter of fact, frequencies at the latter sites were almost as high as for chondrocytes.

Figure 3 provides also interesting information about the relative frequencies of Helix-II, CTX-II and PIIANP immunoreactivity. Overall, Helix-II is detected the most frequently and CTX-II the less. Actually in most positions, Helix-II is detected at least twice as frequently as CTX-II. However, Helix-II was never detected in calcified matrix

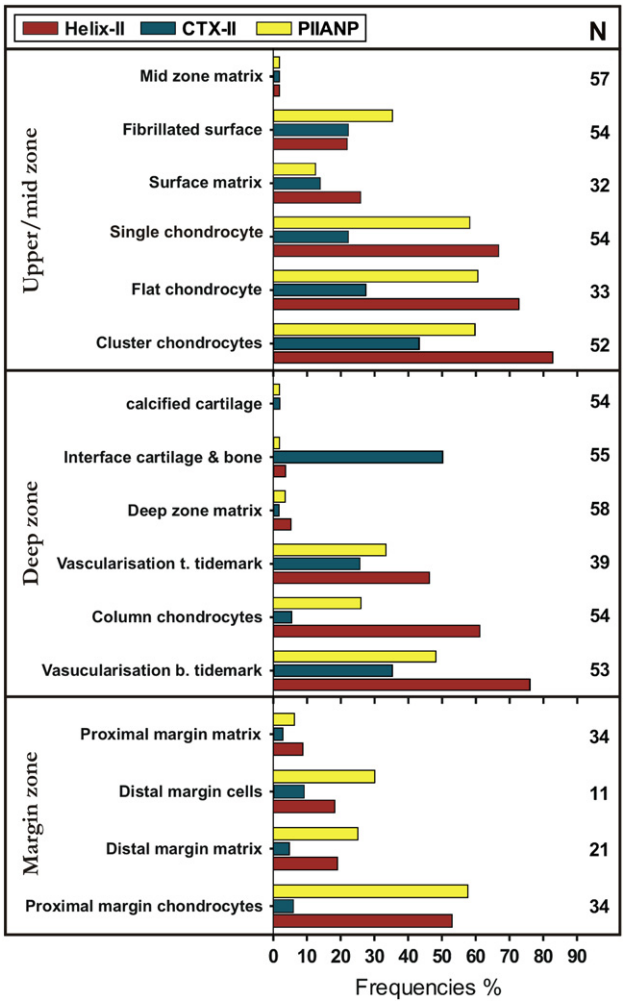


Fig. 3. Frequency of association of marker immunoreactivity with specific histological features. Adjacent sections were obtained from each of the 58 biopsies, and immunostained for Helix-II, CTX-II, and PIIANP. The distribution of these immunoreactivities was analyzed, paying attention to the histological features listed in the graph, and which are shown in three distinct groups according to the cartilage zone where they are localized. The number of biopsies where these features were identified is shown in the right column (*N*). The number of biopsies showing an association of the respective markers with each of these features was normalized to *N*, and is shown by the length of the bars. For convenience of comparison, the features were ranked within each of cartilage zones, according to increasing frequency of association with Helix-II.

while CTX-II was detected at this level. Furthermore, CTX-II was the prevailing marker at the bone–cartilage interface (positive in about 50% of the biopsies), and was as frequent as Helix-II at fibrillated surfaces. Note also the peculiarity of the margin zone showing rather high frequency of PIIANP relative to Helix-II, and rarely CTX-II.

### ANALYSIS OF THE DISTRIBUTION OF COLLAGEN TURNOVER MARKERS IN RELATION WITH THE CHARACTERISTICS OF THE INDIVIDUAL BIOPSIES

The analysis of Fig. 4 shows that the extent of immunoreactivity through the cartilage tissue was variable, depending on which biopsy and on which marker is considered. Two biopsies were completely negative for Helix-II, 16 for CTX-II and five for PIIANP, but all the biopsies showed immunoreactivity for at least one marker, and when immunoreactivity was wide-spread, up to 75%, 50%, and 64% of the analyzed features were positive for Helix-II, CTX-II, and PIIANP, respectively. The bone origin (femur condyles or tibia plateau) or joint area (lateral or median) neither affected the extent nor the position of the immunoreactivity, whatever the marker. Similarly, the Mankin score in the 5–10 range did not correlate with the degree of spreading, but it is remarkable that the deep zone matrix areas that were positive for Helix-II or CTX-II or PIIANP, which were only very few, all showed Mankin score 10. Figure 4 also shows that there is no strict correlation between Helix-II, CTX-II and PIIANP with respect to extent of spreading. For example, there are biopsies showing at least five positive Helix-II features, but no CTX-II at all. There is, however, no evidence that the spreading of Helix-II and CTX-II varies in opposite directions, which would have meant that different proteolytic mechanisms prevail in different biopsies. Interestingly, when distribution of immunoreactivity is restricted to one or two features, the three most frequent ones included clustered chondrocytes of the upper/midzone and vascularization below the tidemark, whatever the marker. The third prevailing feature was the cartilage–bone interface for CTX-II and single chondrocytes for Helix-II and PIIANP. In situations where distribution of immunoreactivity extended to five features or more, the prevailing positive features included the same as in the biopsies with restricted immunostaining, plus the flat chondrocytes of the upper zone, the chondrocytes of the margin, and the chondrocytes of the deep zone for Helix-II and PIIANP. Thus this analysis indicates that spreading of immunoreactivity of each of the markers to more features occurs to some degree in an ordered way, which seems mainly determined by the intrinsic properties of these features. It also indicates that amongst these features, some are mainly characterized by a single marker (e.g., bone–cartilage interface for CTX-II); a few show a parallel behavior for only Helix-II and PIIANP (e.g., the flat and single chondrocytes of the upper/midzone, chondrocytes of the margin zone, and column chondrocytes of the deep zone); and a few show a parallel behavior for the three markers (e.g., vascularization and clustered chondrocytes).

### CO-LOCALIZATION OF COLLAGEN TURNOVER MARKERS AT THE LEVEL OF GIVEN HISTOLOGICAL FEATURES

Because of the parallel behavior of some of the markers in Figs. 3 and 4, we analyzed which showed statistically significant co-localizations and which features were concerned by these co-localizations (Table I). Significant co-localizations were found only for a few features. These included the vascularization sites for the three markers taken 2 by 2; the single chondrocytes for Helix-II/PIIANP and Helix-II/CTX-II; column chondrocytes for Helix-II/PIIANP, and flat chondrocytes for Helix-II/CTX-II. In contrast, other positions that appeared frequently positive for two or three markers were most often not simultaneously positive for these markers. This analysis thus suggests that collagen deposition and breakdown into Helix-II and CTX-II are simultaneous at the level of a few well-defined features, but do not occur simultaneously at the level of most features.

## Discussion

The present study demonstrates the association of type II collagen breakdown and synthesis markers with specific

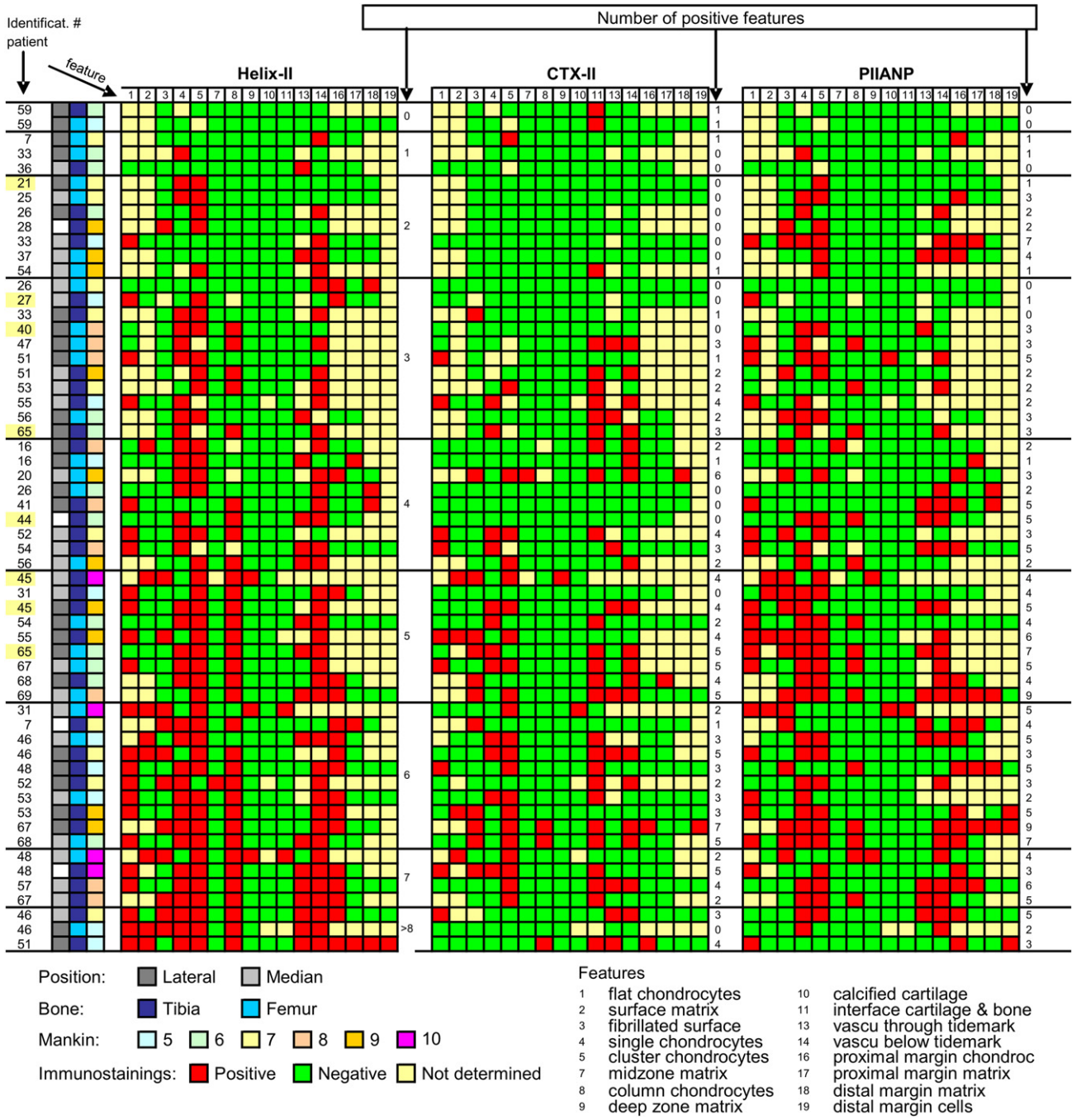


Fig. 4. Distribution of marker immunoreactivity in each biopsy. The data used for the analysis of Fig. 3 were organized in a table where each line represents a biopsy, each column represents a feature probed for Helix-II, CTX-II, or PIIANP in three adjacent sections, and each cell indicates whether immunoreactivity is present (red), absent (green), or could not be detected because the feature was absent in this particular section (yellow). The number of positive features in each section is shown for the respective markers, and the biopsies were ranked according to increased spreading of Helix-II immunoreactivity. The three columns to the left indicate the site of origin of the section within the joint (tibia or femur and lateral or median), as well as the Mankin score assessed in adjacent sections. Patients not diagnosed for primary OA are shown with an identification number on a yellow background in the left column.

histological features of OA cartilage. These associations are especially interesting to discuss taking into account the specificities of these markers for defined collagen breakdown products, and the response of their body fluid levels to disease situations. Also some of the methodological aspects used here are unique compared with previous studies. Of note is (1) the simultaneous assessment of two breakdown products and one synthesis marker, as well as histological features in adjacent sections, thereby allowing direct comparisons of the association of the markers with single histological features; and (2) separate quantifications of the frequency of these associations for each of these histological features. This approach allowed avoiding the "noise" generated by the heterogeneity of

Table I
*Co-localization of Helix-II, CTX-II, and PIIANP*

| | Helix-II vs CTX-II | | Helix-II vs PIIANP | | CTX-II vs PIIANP | |
|---|---|---|---|---|---|---|
| | *P* | *N* | *P* | *N* | *P* | *N* |
| Midzone matrix | 1 | 57 | 1 | 57 | 1 | 57 |
| Fibrillated surface | 0.227 | 54 | 0.087 | 54 | 0.018* | 53 |
| Superficial matrix | 0.300 | 29 | 0.269 | 31 | 0.298 | 28 |
| Single chondrocytes | 0.009** | 53 | 0.019* | 54 | 0.051 | 54 |
| Flat chondrocytes | 0.039* | 33 | 0.107 | 33 | 0.667 | 31 |
| Clusters | 0.060 | 51 | 0.457 | 52 | 1.000 | 47 |
| Calcified cartilage | – | 54 | – | 54 | 0.037* | 55 |
| Interface cartilage and bone | 0.236 | 55 | 0.036* | 55 | 0.491 | 55 |
| Deep zone matrix | 0.052 | 58 | 0.002** | 58 | 0036* | 58 |
| Vascularization through tidemark | 0.014* | 37 | 0.038* | 37 | 0.045* | 37 |
| Column chondrocytes | 0.274 | 54 | 0.0003*** | 54 | 0.161 | 54 |
| Vascularization below tidemark | 0.021* | 54 | 0.0004*** | 52 | 0.149 | 53 |
| Proximal margin matrix | 0.912 | 34 | 0.113 | 32 | 0.781 | 32 |
| Distal margin cells | 0.182 | 11 | 0.067 | 10 | 0.300 | 10 |
| Distal margin matrix | 0.810 | 21 | 0249 | 20 | 0.730 | 20 |
| Proximal margin chondrocytes | 0.273 | 34 | 0.114 | 33 | 0.324 | 33 |

The data of Fig. 4 were analyzed for co-localization of the collagen turnover markers with a given feature, by using Fisher's exact test. The columns show the *P* values for the co-localizations of the markers taken 2 by 2, and the number of biopsies involved in this evaluation. Statistical significant co-localizations: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

OA cartilage and distinguishing clear patterns of localization for each of the markers.

Collagen breakdown in cartilage sections was previously assessed only with probes detecting collagenase-initiated cleavage in the triple helix, and they showed prevailing signals at the articular surface and around chondrocytes, extending sometimes to the interterritorial matrix, including deep zone cartilage[4–6]. In the present study we probed cartilage sections with two different probes for collagen fragments: one recognizing a fragment originating from the type II collagen triple helix like in previous studies although from a different region, and one originating from the telopeptide region of type II collagen for comparison[14]. These two probes stained cartilage surface including fibrillated areas and chondrocytes, just like did the probes used in previous studies. However, frequently stained areas included also regions toward the subchondral bone where collagen fragmentation signals were not reported previously. This observation is compatible with the possible role of subchondral bone in OA[8,9]. For instance, vascularization sites in the deep zone cartilage were frequently positive for Helix-II and CTX-II, even in sections showing restricted immunostaining. Furthermore, the cartilage–bone interface stained for CTX-II, not Helix-II, and this was actually CTX-II's prevailing position, also when immunostaining was restricted. Also the marginal area showed collagen breakdown, but in contrast, mostly through Helix-II and not so much through CTX-II.

It is remarkable that our analysis shows a series of positions where CTX-II is frequently detected and where Helix-II is absent or almost, and *vice versa*. This absence of spatial correlation between Helix-II and CTX-II in cartilage tissue may relate to the fact that the body fluid levels of Helix-II and CTX-II do not correlate well with each other despite both are elevated in OA patients[14,29]. Noteworthy, as suspected by O'Kane *et al.*[42], CTX-II prevailed compared to Helix-II, in areas close to the subchondral bone, such as the bone–cartilage interface or the mineralized matrix. The latter may relate to the unique response of CTX-II to agents/situation affecting bone turnover[23,25–27]. This includes also the menopause status which correlated with increased CTX-II[19,43] but decreased C2C, a helix epitope[2], and which did not show any significant correlation with Helix-II[28]. The actual mechanism at the origin of these differences in Helix-II and CTX-II tissue localizations is unclear. As a matter of fact Helix-II and CTX-II originate from collagen domains known for their different susceptibility to proteolytic attack[14], and test tube experiments have demonstrated that the proteolytic pathways determining the generation of Helix-II and CTX-II involve very different proteinases[13]. Thus if different proteinases prevail in cartilage and bone, one may expect different fragmentation patterns at the bone–cartilage interface compared to further inside the cartilage. One should also be aware that the respective positions of Helix-II and CTX-II do not necessarily coincide with the sites of their generation. They may also relate to the relative binding strength of these fragments to given histological features while diffusing away from their site of origin. As a matter of fact Helix-II and CTX-II should diffuse readily away from cartilage since their levels in the body fluids of OA patients proved to be elevated[14]. This may explain why they generated low matrix staining. This is in contrast with previous studies detecting damaged collagen appearing often retained in the tissue[5,6,44]. The different position of Helix-II and CTX-II signals may also relate to differences in susceptibility to destruction. For instance, cathepsin B is known to degrade Helix-II[13] so that the absence of Helix-II at the bone–cartilage interface could be ascribed to the possible high levels of cathepsin B in these areas, by analogy with high levels of cathepsin B at the bone–cartilage interface of the growth plate[45].

It is also interesting that the chondrocytes are the most frequently stained feature. Since they are also the site of type II collagen and proteinase synthesis, one may wonder whether Helix-II and CTX-II originate partially from newly synthesized collagen that is rapidly degraded rather than from the degradation of the matrix collagen network. This hypothesis was put forward earlier[11] and did not prove to be supported by assessments of collagen synthesis and breakdown performed in extracts of full-depth cartilage[46]. Because such extracts contain very diverse cartilage tissue that may generate noise and obscure very localized correlations,

this hypothesis was reanalyzed in the present study by assessing the immunoreactivity of the fetal propeptide of type II collagen, PIIANP, separately at the level of different features and comparing it with Helix-II and CTX-II immunoreactivity at the level of the same features in the adjacent section. Our analysis showed similar localization of PIIANP as in previous propeptide immunolocalization studies[31,32] (chondrocytes of middle zone and association with the matrix in the upper zone and deep zone on some occasions), but also at sites that were not reported previously, such as vascularization sites and margin zone. Actually, the latter two sites together with chondrocytes were those where PIIANP was most frequently detected, as is also the case for Helix-II. When analyzing co-localizations statistically, significant correlations between Helix-II and PIIANP were seen for most of the features of the deep zone, including column chondrocytes, vascularization sites, and deep zone matrix. There was also significant co-localization between CTX-II and PIIANP at the level of some features although to a lower extent. These co-localizations suggest that Helix-II and CTX-II may reflect partially degradation of newly synthesized collagen at some sites. However, for many features of other cartilage regions, there was no significant co-localization of Helix-II and PIIANP and even less so for CTX-II and PIIANP, which may explain the absence of correlation between CTX-II and PIIANP levels in body fluids[31,33,34], as well as the absence of correlation between PIICP and collagen breakdown in extracts of full-cartilage sections[46].

Collagen turnover markers and Mankin scores correlated in some studies but not in others[5,44,46]. Our study did not show correlations, although the probes we used were able to detect broader collagenolytic actions than previous studies. Absence of correlations may be due to the fact that the Mankin scores of our biopsies were within a too narrow range. It may also be due to the fact that the Mankin scale scores final damage that cannot be directly compared with the early molecular alterations detected with the collagen turnover markers. Furthermore, our analysis stresses that the response of chondrocytes to the OA condition was not random as often suggested by the heterogeneity of OA cartilage, but proceeded in an ordered sequence, both for breakdown and synthesis markers. In biopsies with restricted stainings, the positive chondrocytes were clustered chondrocytes of the upper/middle zone. Upon spreading of immunoreactivity, flat chondrocytes close to the surface become also positive, and Helix-II and PIIANP become also associated with chondrocytes of the deep zone and the marginal zone. Thus it is in a highly orchestrated way that the chondrocytes switch to a high collagen turnover phenotype.

One could wonder whether the present immunostaining patterns reflect the biological reality, or result merely from technical peculiarities, arguing for instance that the chondrocytes are the most frequently stained features because of the better penetration of the antibodies at this level. However, as shown in Table I and discussed above, even cartilage features that are frequently immunostained by two different markers like chondrocytes show only rarely simultaneous localizations of these two different markers in the same biopsy. Thus the factor determining which of two markers immunostains a feature does not relate to the intrinsic accessibility of this feature to an antibody, but is biopsy dependent. It is also of interest to mention that similar immunostaining patterns were obtained in cryosections (not shown).

In conclusion, two collagen breakdown probes that were elevated in body fluids of OA patients could be detected in OA cartilage tissue, thereby providing evidence for their relevance to cartilage tissue events. These probes show signs of collagen breakdown at the articular surface and at the level of chondrocytes as previously detected with collagenase specific probes, but also at vascular sites and bone–cartilage interface where collagen damage was not detected previously. These latter sites were even amongst the first to be stained when ranking the tissues for increased spreading of staining. These newly detected locations are compatible with the suspected role of bone–cartilage interactions in OA. These detections may be ascribed to the fact that we used probes recognizing fragments generated by proteinases with different cleavage specificities, thereby allowing the detection of broader distributions. Finally, because of the limited co-localization of PIIANP with collagen breakdown products, our study supports the view that collagen breakdown products result only partially from newly synthesized collagen (i.e., mainly in the deep zone). It remains, however, to be investigated whether there is a direct correlation between a specific immunostaining pattern in given knees and the level of CTX-II or Helix-II in the body fluids of the corresponding OA patient, as well as how the immunoreactivity would appear in cartilage from a healthy patient.

Overall our study supports the use of Helix-II, CTX-II and PIIANP levels in body fluids as markers for cartilage collagen turnover in OA. It also supports the hypothesis that combined measurements of several markers may be required for evaluating appropriately the damage in cartilage, but the actual demonstration of this hypothesis still requires a clinical study addressing directly this question.

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